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Morphine-induced MOR-1X and ASF/SF2 expression is independent of transcriptional regulation: Implications for MOR-1X signaling

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Abstract

Recently, multiple μ -opioid receptor (MOR) isoforms have been identified that originate from a single gene, OPRM1; however, both their regulation and their functional significance are poorly characterized. The objectives of this study were to decipher, first, the regulation of alternatively spliced μ -opioid receptor isoforms and the spliceosome components that determine splicing specificity and, second, the signaling pathways utilized by particular isoforms both constitutively and following agonist binding. Our studies demonstrated that the expression of a particular splice variant, MOR-1X, was up-regulated by morphine, and this coincided with an increase in the essential splicing factor ASF/SF2. Structural comparison of this isoform to the prototypical variant MOR-1 revealed that the unique distal portion of the C-terminal domain contains additional phosphorylation sites, while functional comparison found distinct signaling differences, particularly in the ERK and p90 RSK pathways. Additionally, MOR-1X expression significantly reduced Bax expression and mitochondrial dehydrogenase activity, suggesting a unique functional consequence for MOR-1X specific signaling. Collectively, these findings suggest that alternative splicing of the MOR is altered by exogenous opioids, such as morphine, and that individual isoforms, such as MOR-1X, mediate unique signal transduction with distinct functional consequence. Furthermore, we have identified for the first time a potential mechanism that involves the essential splicing factor ASF/SF2 through which morphine regulates splicing specificity of the MOR encoding gene, OPRM1.

INTRODUCTION

Since the discovery of the first opioid receptor in 1973, four opioid receptors subtypes have been identified, among which the μ -opioid receptor (MOR), encoded by a gene located on human chromosome 6 now known as the OPRM1 (Kaufman et al., 1995; Wei and Loh, 2011), represents the most clinically relevant as it has a high affinity for classical opioid agonists, such as morphine and heroin, and antagonists, such as naloxone. Its biological significance can be inferred from the fact that it is highly conserved across species, with more than 95% homology between the human and rat receptors (Abbadie and Pasternak,

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2002; Wang et al., 1994). Despite the identification of four opioid receptor subtypes, a four opioid receptor model does not readily predict the clinical observations of opioid pharmacology. For example, gene knockouts targeting exon 1 of the OPRM1 gene are sufficient to abolish analgesia mediated by the μ -selective agonist morphine; however, they are insufficient to abolish analgesia mediated by the morphine metabolite M6G or by heroin (Kieffer, 1999; Schuller et al., 1999). Multiple studies investigating opioid pharmacology have suggested the existence of additional opioid receptors but, although additional receptors have been proposed, evidence in support of the existence of additional opioid receptor types is lacking given that no additional opioid receptor-encoding genes have been identified. Instead, studies have shown that transcriptional and post-translational modifications of opioid receptors are involved in the diverse pharmacology observed with opioid agonists. Of these regulatory mechanisms, alternative splicing is particularly interesting given that it results in the synthesis of multiple, structurally different proteins from an individual gene. This is particularly true for the MOR, which exhibits the most complex and extensive splicing patterns among classical opioid receptors (Chevlen, 2003; Doyle et al., 2007; Kvam et al., 2004; Mayer et al., 1996; Mizoguchi et al., 2003; Pan, 2003; Pan et al., 1999; Pan et al., 2001; Pan et al., 2003; Pasternak, 2001; Pasternak, 2014; Pasternak and Pan, 2013; Xu et al., 2014). Given that MOR isoforms exhibit unique cellular and subcellular localization, ligand binding, cell signaling, desensitization, internalization, and recycling characteristics (Markovic and Challiss, 2009; Milligan, 2003; Wong, 2003), each MOR isoform must be regarded as a separate receptor subtype that collectively contributes to the overall cellular and physiological effects of opioids. Therefore, alterations in the MOR isoform profile may alter the balance within this collective signaling, thereby altering opioid pharmacology. Although it is well-known that the physiological and cellular response to opioids is altered by numerous factors, most notably prolonged clinical use and abuse of opioids, through the modulation of opioid receptor expression and the establishment of opioid tolerance (Dang et al., 2011; Schmid and Bohn, 2009), mechanisms that regulate MOR splicing specificity are poorly understood, as are extracellular factors that alter MOR splicing patterns and the functional significance of shifting isoform expression profiles. Recently, it has been suggested that opioid use may promote changes in MOR splicing patterns, as individuals maintained on methadone exhibit altered expression of certain splice variants (Vousooghi et al., 2009). Whether this is a direct result of methadone treatment, prior substance abuse, or representative of a genetic predisposition for the development of opioid addiction is still unknown; however, limited studies have suggested that chronic morphine treatment can alter MOR splicing through a yet unknown mechanism (Verzillo et al., 2014; Xu et al., 2015). Therefore, this study set out to first establish whether morphine treatment directly impacts the alternative splicing of the MOR and the mechanism through which this is mediated. Second, this study examined the unique signaling cascades activated by those MOR isoforms identified to be regulated by morphine in order to assess the cellular consequences of morphine-mediated changes in MOR alternative splicing patterns in opioid pharmacology.

MATERIALS AND METHODS

Cell Culture

The human neuroblastoma cell line SH-SY5Y was obtained from the American Type Culture Collection (ATCC) and was maintained in Neurobasal media supplemented with B-27 supplement (Invitrogen), 2mM GlutaMAX (Invitrogen), and 50µg/mL gentamicin (Invitrogen) in a humidified incubator at 37°C with 7% CO₂. Cells were split and subcultured using 0.25% Trypsin-EDTA solution and subcultures were not maintained after 20 passages. The human embryonic kidney cell line HEK293 was also obtained from the ATCC and was maintained in Dulbecco's Modified Eagle's Medium (DMEM) and supplemented with 2% heat-inactivated fetal bovine serum (FBS) and 50µg/mL gentamicin in a humidified incubator at 37°C with 7% CO₂. For experiments, SH-SY5Y cells were plated in uncoated 6-well plates at a confluence of 5×10^5 cells/well and incubated for 24 hours to allow for attachment before treatment. HEK293 cells were serum starved for 24 hours using DMEM supplemented with 0.2% FBS and 50µg/mL gentamicin prior to experiment. Cells were then plated on poly-d-lysine coated 6-well or 12-well plates at a confluence of 2×10^5 cells/well or 1×10^5 cells/well, respectively, and were incubated for 36 hours to allow for attachment before treatment.

Plasmid Constructs & Transfection

The pCMV6-MOR-1 and pCMV6-MOR-1X expression plasmids, which transcribe Myc/DDK-tagged, full-length MOR-1 and MOR-1X, respectively, were purchased from OriGene. The CAT expression constructs pBLCAT3-pSF2 (−400 to +47) and pBLCAT3-pSF2 (−200 to +47) were generated previously by our lab (Sariyer, 2010). The pCGT7-empty vector plasmid was used as a control for transfection conditions. Plasmid stocks of all constructs were generated by transforming competent E. Coli DH5α cells and purifying plasmids constructs using the MaxiPrep kit purchased from Qiagen. Purified plasmid constructs were resuspended in DNase/RNase-free dH₂O and stored at −20°C. For experiments, SH-SY5Y and HEK293 cells were transfected with 3µg plasmid DNA using Eugene 6 transfection reagent in a 1:3 and 1:2 ratio, respectively. Briefly, transfection samples were mixed in 100µL of OptiMEM and incubated for 45 minutes at room temperature. Culture media was then aspirated from plated cells and replaced with 100µL transfection sample and 900µL of additional OptiMEM. Following a 4 hour incubation, fresh culture media was added directly to transfection samples and cells were incubated for 48 hours to allow for vector expression.

Morphine and Stress Inducer Treatments

Purified morphine sulfate salt pentahydrate was purchased from Sigma-Aldrich, resuspended in dH₂O to generate a 0.1mM stock solution, and stored at room temperature. Control conditions consisted of serum starvation using Hank's Balanced Salt Solution (HBSS), serum starvation using HBSS with concomitant bafilomycin (Baf) treatment, and treatment with CdCl₂. Stock solutions for Baf and CdCl₂ were created by diluting each into dH₂O to a concentration of 1.5µM and 600mM, respectively. Bafilomycin stock solution aliquots were stored at −20°C, while the CdCl₂ stock solution was stored at 4°C. For final treatment concentrations, morphine (final concentration of 0.1µM, 1µM, or 10µM), and

CdCl₂ (final concentration of 1mM) stock solutions were diluted into culture media directly, whereas bafilomycin was diluted into HBSS to a final concentration of 5nM and added to cells following the removal of culture media. Treatment courses in SH-SY5Y and HEK293 cell lines were conducted in such a way that all experimental conditions were harvested at the same time point. For chronic morphine treatments, the morphine stock solution was diluted directly into culture media every 2 hours for 24 hours. At the 20 hour time point, bafilomycin and HBSS was added to create a 4 hour starvation condition. At 23 hours, the CdCl₂ stock solution was added to create a 1 hour apoptotic condition. All treatments were harvested at 24 hours.

Extraction and Purification of Cytoplasmic RNA

Cytoplasmic RNA was extracted from treated SH-SY5Y cells using the RNEasy Mini Kit (Qiagen) following the manufacturer's protocol adjusted for purification of cytoplasmic RNA fractions only. Briefly, treated cells were trypsinized, transferred to RNase-free micro-centrifuge tubes, and centrifuged at 8,000rpm for 5 minutes. Media was aspirated and the cell pellet was washed with 1mL ice-cold PBS. Washed cells were centrifuged again then lysed in complete RLN buffer (50mM Tris-HCl pH 8, 140mM NaCl, 1.5mM MgCl₂, 0.5% NP-40, 1U/μL RNase Inhibitor (Invitrogen), and 1mM DTT) for 5 minutes on ice. The cytoplasmic RNA-containing fraction was then separated from the nuclear and plasma membrane fractions by centrifugation at 8,000rpm for 5 minutes and cytoplasmic RNA-containing supernatant was transferred to fresh, RNase-free micro-centrifuge tubes. Cytoplasmic RNA samples continued to be processed following the Qiagen RNEasy protocol and purified cytoplasmic RNA was resuspended in RNase-free dH₂O. DNA contamination was removed by treating cytoplasmic RNA samples with RNase-free recombinant DNase I (Roche) following an optimized protocol. Briefly, cytoplasmic RNA samples were resuspended to a volume 50μL with RNase-free dH₂O. DNase treatment samples were prepared by adding 5.5μL 10x DNase Buffer and 2μL recombinant DNase I and incubated at room temperature for 15 minutes. Samples were then treated with 25mM EDTA, incubated at 65°C for 10 minutes, and placed immediately on ice. Prior to conducting two-step RT-PCR experiments, cytoplasmic RNA was repurified in order to remove DNase enzymes and buffer salts. DNase treated samples were increased to a volume of 200μL using RNase-free dH₂O, subjected to purification via phenol/chloroform extraction, and precipitated using ethanol. Precipitated, DNase-treated cytoplasmic RNA was then resuspended in RNase-free dH₂O to a concentration of 300ng/μL and stored at -70°C.

Primer Design, Semi-Quantitative Two-Step RT-PCR, and Gel Electrophoresis

Semi-quantitative, two-step RT-PCR was conducted using High Fidelity Platinum Taq and Superscript III Reverse Transcriptase (RT) enzymes following optimized protocols. Initial RNA denaturation and priming samples (100ng sample RNA, 0.5μg/μL OligodT₁₂₋₁₈ Primer, and 2x reaction mix) were added to thin walled, RNase-free PCR tube and incubated at 65°C for 5 minutes then placed immediately on ice. Samples were then transferred to fresh, thin walled, RNase-free PCR tubes containing additional 2x reaction buffer and 1U/μL Superscript III RT. Synthesis of complementary DNA (cDNA) was then initiated by incubating the samples at 50°C for 50 minutes, then 85°C for 5 minutes before being placed

on ice. cDNA samples were transferred to fresh, thin-walled, RNase-free PCR tubes containing an equal volume of PCR master mix (1x High Fidelity Buffer, 0.4mM dNTP mix, 2mM MgSO₄, 1.5U/ μ L High Fidelity Platinum Taq, RNase-free dH₂O) and 0.2 μ M of both forward and reverse primers. Primers were ASF/SF2 forward 5'-ACC TTC CAT CTA GAT CGG GAG GTG GTG TGA TTC GT-3' and reverse 5'-TTC CAG GAT CCT TAG TCG CGA CCA TAC ACC GCG TCT T-3', MOR-1 forward 5'-TGC TCA GCT CGG TCC CCT CC-3' and reverse 5'-GCA GAG CAG AGT GGC CAG AG AG-3', MOR-1X forward 5'-TGC GCC TCA AGA GTG TCC GC-3' and reverse 5'-CTC CAC CAG ACG GGC TGG GA-3', and GAPDH ReadyMade forward and reverse primers purchased from Integrated DNA Technologies (IDT). Samples were then placed in a PCR cycler, initially denatured at 94°C for 2 minutes, then run for 35 cycles of 30 seconds at 94°C, 30 seconds at an optimized annealing/extension temperature of 65°C, and 3 minutes at 68°C. Following PCR cycling, a final extension was conducted at 68°C for 10 minutes then samples were cooled to 4°C.

Gel electrophoresis was used to determine PCR amplification. Electrophoresis-grade agarose powder was dissolved in 1x TAE buffer (40mM Tris, 20mM acetic acid, 1mM EDTA) with 50ng/mL ethidium bromide to create a 2% agarose gel. Electrophoresis samples were prepared by adding 25 μ L of PCR samples to 5 μ L 6x DNA loading buffer (30% glycerol, 0.25% bromophenol blue in 1x TAE buffer). Samples were loaded and run at 75V for 1 hour and visualized using the Kodiak Gel Logic Imaging System. Semi-quantitative analysis was performed using the ImageJ imaging software (Schneider et al., 2012).

Extraction and Purification of Whole Cell Proteins

Whole cell protein lysates were extracted from SH-SY5Y and HEK293 cells following an optimized protocol from Abcam. Briefly, treatment media was aspirated from culture wells and plated cells were washed once with ice-cold PBS. Cells were then lysed directly in culture wells using ice-cold RIPA lysis buffer (150mM NaCl, 50mM Tris-HCL pH 8, 1% NP-40, 0.5% Na Deoxycholate, 0.1% SDS) supplemented with respective serine/threonine and tyrosine phosphatase inhibitors NaF (10mM) and Na₃VO₄ (1mM) as well as a 1x mammalian protease inhibitor cocktail purchased from Sigma-Aldrich. Cells were rocked in complete RIPA lysis buffer for 5 minutes at 4°C before being scraped from the well, transferred to micro-centrifuge tubes, and rotated at 4°C for an additional 30 minutes. Protein lysates were centrifuged at 13,000rpm for 5 minutes and supernatant was transferred to fresh micro-centrifuge tubes. Protein concentrations were determined using the Bio-Rad Bradford Reagent and a 96-well plate reader. Purified protein lysates were stored at -20°C.

Discontinuous Electrophoresis and Li-Cor Western Blotting Analysis

Equal concentrations of protein lysates were mixed with 6x Laemmli Buffer (12% SDS, 30% β -mercaptoethanol, 60% glycerol, 375mM Tris HCl pH 6.8) and RIPA lysis buffer to bring all samples to a total volume of 50 μ L. Bromophenol blue was not added to the loading buffer as it interferes with fluorescent analysis using the Li-Cor imaging system. Samples were then denatured at 100°C for 5 minutes, vortexed, centrifuged at 13,000rpm, and loaded into wells of a 12% SDS-polyacrylamide gel for separation by SDS-PAGE. Discontinuous electrophoresis was performed at 200V for 1 hour using a Tris-Glycine-SDS (TGS) buffer

(25mM Tris-Base, 250mM Glycine, 3.5mM SDS). Proteins were transferred onto methanol-activated, Li-Cor-specific PVDF membranes using a wet transfer system containing Tris-Glycine transfer buffer (26.5mM Tris-Base, 192mM Glycine, 1% methanol) and run at 250mA for 3 hours. Membranes were dried at room temperature and stored protected from light.

Quantitative analysis using the Li-Cor Odyssey CLx Infrared Imaging System was performed following an optimized protocol. Briefly, dried PVDF membranes were reactivated in methanol and washed in PBS. Membranes were then blocked for 1 hour, with shaking, in a 5% milk:PBS solution. Primary monoclonal and polyclonal antibodies against ASF/SF2 (Invitrogen), β -tubulin (Sigma-Aldrich), Beclin-1 (Cell Signaling), LC3 (Sigma-Aldrich), and Bax (Cell Signaling) were diluted 1:1000 in a 5% milk:PBS solution as well. For fluorescence analysis of proteins, primary antibodies were added to membranes and probed, with shaking, for either 1–2 hours at room temperature or overnight at 4°C. The primary antibody solutions were then removed and membranes were extensively washed with PBS containing 0.1% Tween-20 three separate times consecutively for 5 minutes each, removing wash buffer between washes, with a final wash of PBS without Tween-20 for 5 minutes. Secondary antibody dilutions of 1:5,000 were prepared in a 5% milk:PBS solution by adding Li-Cor-specific IRDye 800CW goat anti-mouse IgG and/or IRDye 680CW goat anti-rabbit IgG. Membranes were probed with secondary antibodies for 1 hour at room temperature, protected from light, then extensively washed with PBS containing 0.1% Tween-20 as before, followed by a final wash of PBS to remove residual Tween-20. Membranes were then scanned and analyzed using the Li-Cor Odyssey CLx Infrared Imager (LI-COR; Millennium Science, Surrey Hills, Australia).

MAPK Array Analysis

Total cell lysates were prepared from HEK293 cells transfected with plasmids expressing either MOR-1 or MOR-1X, which were left untreated or treated with morphine, using Human Phospho-MAPK Array Kit (R&D Systems, Minneapolis, MN) lysis buffer following the manufacturer's protocol. Protein concentrations were determined using the Bio-Rad Bradford Reagent and a 96-well plate reader. Manufacturer-supplied membranes were blocked with Array buffer 5 for 1 hour at room temperature. Array buffer 5 was then aspirated and membranes were rocked overnight at 4°C in a mixture of the manufacturer-supplied antibody mix and 200 μ g fresh protein lysates adjusted to a final volume of 1.5 mL with Array Buffer 1. The membranes were then processed using a manufacturer-supplied chemo-luminescent solution according to the manufacturer's protocol. Probed dot blots were exposed on radiographic film, which were developed and scanned for semi-quantitative analysis using ImageJ software.

CAT Reporter Gene Analysis

Activity of the ASF/SF2 encoding gene, SFRS1, was determined using a CAT reporter gene assay following an optimized protocol. Briefly, treated pBLCAT3-pSF2 (–400 to +47) or pBLCAT3-pSF2 (–200 to +47) expressing SH-SY5Y cells were washed twice with PBS then incubated in TEN Buffer (40mM Tris HCl pH 7.5, 1mM EDTA pH 8, 150mM NaCl) for 5 minutes at room temperature with shaking. Cells were removed by scraping,

transferred to micro-centrifuge tubes, and centrifuged at 13,000rpm and 4°C for 1 minute. TEN buffer supernatant was aspirated from samples and pelleted cells were resuspended in ice-cold 0.25M Tris HCL pH 7.5. Lysis of resuspended cells was performed using three freeze/thaw cycles and lysates were allowed to cool for 10 minutes on ice. Samples were centrifuged at 13,000rpm and 4°C for 5 minutes and the protein-containing supernatants were transferred to fresh micro-centrifuge tubes. CAT reporter gene assay samples were prepared using 30µg protein and incubated at 37°C for 1 hour. Samples were then mixed with ethyl acetate, lyophilized, resuspended in 18µL of ethyl acetate, and placed drop-wise onto chromatography paper. Liquid chromatography was then performed using a 19:1 chloroform/methanol running buffer. Exposures of chromatography paper were conducted at -70°C for 12 hours protected from light. Liquid scintillation counting was used to quantitate CAT reporter gene expression.

MTT and Viability Assays

Mitochondrial dehydrogenase activity was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay following an optimized protocol. Briefly, MTT solution (5mg/mL MTT in PBS) was added directly to the culture media of treated HEK293 cells plated in 12-well culture plates. Cells were incubated at 37°C for 1 hour before the MTT-containing culture media was aspirated. The resulting formazan within MTT-treated cells was solubilized by the addition of an MTT solvent (4mM HCl, 0.1% NP-40 in 2-propanol). Solubilized formazan samples were then transferred to a 96-well plate and absorbance was measured at 590nm, using absorbance at 620nm as a reference. Early and late apoptosis was determined by Annexin/7-AAD staining and cell sorting using a Guava flow cytometer and Guava Nexin Kit following the manufacture's protocol and associated software (Millipore).

RESULTS

Morphine increases MOR-1X mRNA expression in SHSY-5Y cells

Multiple factors, including methadone and chronic morphine treatment, are correlated with changes in the expression of specific MOR isoforms, particularly within certain CNS tissues (Verzillo et al., 2014; Vosooghi et al., 2009; Xu et al., 2015); however, a direct role for opioids in mediating cell type-specific splicing of the human OPRM1 gene has not been shown. Therefore, using semi-quantitative RT-PCR, this study examined the splicing pattern of the MOR in undifferentiated SH-SY5Y cells, a model for human dopaminergic neurons, both endogenously and following treatment with 0.1µM morphine for either 1 hour or 24 hours. In the absence of morphine, expression of several MOR isoforms was observed, including the prototypical isoform MOR-1 (Figure 1, lane 1); however, the MOR-1X variant was not detected (Figure 1, lane 1). The expression of MOR-1 mRNA was not significantly altered by treatment with 0.1µM morphine for either 1 hour or 24 hours (Figure 1, lanes 2–3). Conversely, mRNA expression of the MOR-1X variant was significantly increased following treatment with 0.1µM morphine for both 1 hour (Figure 1, lane 2) and 24 hours (Figure 1, lane 3).

Morphine-mediated increases in MOR-1X mRNA coincides with increases in the auxiliary splicing factor ASF/SF2 and is independent of transcriptional regulation

The prototypical SR family member protein, ASF/SF2, serves a unique and critical role in splicing and, as such, fluctuations in ASF/SF2 subcellular localization and concentration are sufficient to alter splicing patterns (Wang and Manley, 1995). Expression of ASF/SF2 protein was significantly increased by 1 hour treatments of 0.1 μ M morphine (Figure 2A, lane 2) and 1 μ M morphine (Figure 2A, lane 3), which correlates with both the dosage and timepoint at which morphine significantly increased MOR-1X mRNA expression. The activity of the ASF/SF2 encoding gene, SFRS1, was subsequently examined by CAT assay analysis using transfected pBLCAT3-pSF2 (–400 to +47) and (–200 to +47) reporter constructs to determine if the mechanism through which morphine regulates ASF/SF2 expression involves transcriptional regulation. The activity levels of the –200 or –400 promoter regions of SFRS1 were not significantly altered following a 1 hour treatment with either 0.1 μ M or 1 μ M morphine (Figure 2B). Likewise, there were no significant changes in ASF/SF2 mRNA following morphine treatment (Figure 2C). Therefore, the mechanism through which morphine treatment increases ASF/SF2 protein expression remains unclear but appears to be independent of transcriptional regulation and correlates with increases in MOR-1X mRNA expression.

Comparison of the structural organization and potential functional domains of MOR-1 and MOR-1X suggest differences in receptor phosphorylation

BLAST analysis of the predicted translated protein for MOR-1 and MOR-1X demonstrates over 99% similarity in amino acid sequence. Accordingly, MOR-1 and MOR-1X have identical extracellular N-terminal domains, transmembrane domains, and extracellular and intracellular loops, including a conserved DRY motif in the 2nd intracellular loop, as these regions are transcribed by the conserved exons 1, 2, and 3. Additionally, the proximal portion of the C-terminal domain, which contains a palmitoylated cysteine residue and the agonist-induced phosphorylation motif TXXXPS, expressed as TREHPS and evolutionarily conserved among opioid receptors (Wei et al., 2004), is conserved between the MOR-1 and MOR-1X receptors whereas the distal portion of the C-terminal domain is unique (Figure 3A–B). Given that the C-terminal domains of many GPCRs are the site of phosphorylation reactions essential for initiating conformational changes and GPCR signal transduction, this study inspected predicted phosphorylation motifs within the unique C-terminal domains of MOR-1 and MOR-1X. The unique portion of the MOR-1 C-terminal domain encoded by exon 4 is unremarkable, containing only 12 additional amino acid residues and no additional phosphorylation motifs (Figure 3A), as predicted by the kinase-specific phosphorylation site prediction tool NetPhosK (Blom et al., 2004). Comparatively, the unique portion of the MOR-1X C-terminal domain encoded by exon X is much larger, containing nearly 60 additional amino acid residues and multiple phosphorylation motifs, including 2 serine residues predicted to serve as PKA phosphorylation sites as well as a second copy of the TXXXPS motif, expressed as TAPSPS (Figure 3B). Therefore, despite the two receptors being nearly identical, MOR-1X has a greater potential for phosphorylation than MOR-1 due to the unique portion of the C-terminal domain encoded by the alternative exon X.

Activation of ERK/RSK cascades by MOR-1X is distinct from MOR-1

Opioid agonists are known to stimulate numerous signaling cascades, including MAPK (Gutstein et al., 1997; Schulz et al., 2004). Given that the activation of these pathways is determined primarily by the phosphorylation state of the receptor, and that MOR-1 and MOR-1X show distinct structural differences in their phosphorylation potential due to their unique C-terminal domains, it is possible that MOR-1 and MOR-1X have distinct variations in MAPK pathway activation. Therefore, to identify changes in MAPK signaling of MOR-1 and MOR-1X following activation by morphine, this study examined the signaling of MOR-1 and MOR-1X individually in the HEK293 cell line, which does not endogenously express opioid receptors. HEK293 cells transfected with plasmids expressing either MOR-1 or MOR-1X were assessed using a human Phospho-MAPK array that detects relative phosphorylation of 26 different kinases, including 9 MAPKs. Results showed distinct differences in MAPK signaling in cells expressing MOR-1 and MOR-1X, specifically in the ERK/RSK signaling cascade (Figure 3C–G). Expression levels of phosphorylated ERK1/2 in vector-transfected cells were not prominently affected by treatment with 1 μ M morphine for 15 minutes (Figure 3D, lanes 1–2; Figure 3E, lanes 1–2). However, expression levels of phosphorylated ERK1/2 were increased in both pCMV6-MOR-1 transfected cells (Figure 3D, lane 3–4; Figure 3E, lanes 3–4) and pCMV6-MOR-1X transfected cells (Figure 3D, lane 5–6; Figure 3E, lanes 5–6). Interestingly, pCMV6-MOR-1 transfected cells, but not pCMV6-MOR-1X transfected cells, exhibited a significant response to a 15 minute treatment of 1 μ M morphine, with expression levels of phosphorylated ERK1 increasing and ERK2 decreasing, but still remaining significantly higher than vector-transfected controls (Figure 3D, lanes 3–6; Figure 3E, lanes 3–6).

Expression of phosphorylated p90 RSK1 and 2 (RSK1/2), downstream multi-functional effectors of the ERK1/2 pathway, also exhibited significant differences between pCMV6-MOR-1 and pCMV6-MOR-1X transfected cells. This difference was most prominent for phosphorylated RSK1, as it was significantly increased in pCMV6-MOR-1 transfected cells (Figure 3F, lane 3) but significantly decreased in pCMV6-MOR-1X transfected cells (Figure 3F, lane 5). Furthermore, treatment with 1 μ M morphine for 15 minutes significantly reduced phosphorylated RSK1 expression to baseline levels in pCMV6-MOR-1 transfected cells (Figure 3F, lane 4) but significantly increased its expression above baseline levels in pCMV6-MOR-1X transfected cells (Figure 3F, lane 6). Expression of phosphorylated RSK2 exhibited a similar pattern of inverse regulation, as treatment with 1 μ M morphine for 15 minutes significantly reduced expression below baseline levels in pCMV6-MOR-1 transfected cells (Figure 3G, lane 4) but significantly increased its expression in pCMV6-MOR-1X transfected cells (Figure 3G, lane 6). However, unlike phosphorylated RSK1, expression levels of phosphorylated RSK2 were not significantly altered by the constitutive activity of either MOR-1 or MOR-1X nor were the effects of morphine significantly different from constitutive receptor activity (Figure 3G, lanes 3–6).

MOR-1X uniquely regulates expression of the apoptotic protein Bax but not the autophagic proteins, Beclin-1 and LC3

A well-known substrate of the ERK/RSK pathway is the pro-apoptotic protein Bad, which is inactivated by RSK-mediated phosphorylation, causing it to dissociate from anti-apoptotic

proteins that subsequently inhibit another pro-apoptotic protein, Bax (Anjum and Blenis, 2008; Betito and Cuvillier, 2006; Romeo et al., 2011). Given this role in altering mechanisms of cellular viability and that MOR-1 and MOR-1X alter ERK1/2 and p90 RSK1/2 signaling pathways (Figure 3C–G) in a divergent fashion, this study assessed whether these receptors also exhibit differences in the stimulation of apoptotic signaling cascades, as well as autophagic signaling cascades as this is another common mechanism activated during cellular stress. Investigation of autophagic and apoptotic signaling cascades were conducted using chronic, high dose conditions of morphine established through treatment with 10 μ M morphine every 2 hours for 24 hours as these conditions are often associated with pro-apoptotic signaling cascades mediated through intrinsic and extrinsic apoptotic pathways, whereas both acute administration and chronic, low dose administration are often associated with anti-apoptotic signaling cascades (Chen et al., 2008; Emeterio et al., 2006).

Expression of Bax protein was not significantly altered in untransfected HEK293 cells treated with chronic, high dose morphine (Figure 4A, lane 2), serum starved in HBSS media for 4 hours (Figure 4A, lane 3), or serum starved in HBSS media and treated with 5nM Baf for 4 hours (Figure 4A, lane 4) but was significantly up-regulated in untransfected HEK293 cells treated with 1mM CdCl₂ for 1 hour (Figure 4A, lane 5), a method frequently used for induction of apoptosis through mitochondrial dysfunction. Similarly, Bax protein was not significantly altered in vector-transfected HEK293 cells treated with chronic, high dose morphine (Figure 4A, lane 7). Despite significant effects on phosphorylated ERK1/2 and RSK1/2 expression, the activity of MOR-1 did not differ from the effects observed in MOR-null and vector-transfected HEK293 cells, as Bax protein expression in pCMV6-MOR-1-transfected, untreated cells (Figure 4A, lane 8) and pCMV6-MOR-1-transfected cells treated with chronic, high dose morphine (Figure 4A, lane 9) was not significantly altered. Conversely, Bax expression was constitutively down-regulated in pCMV6-MOR-1X-transfected cells (Figure 4A, lane 10) and was reduced further by chronic, high dose morphine treatment (Figure 4A, lane 11), although the effect of morphine was not significantly different from MOR-1X constitutive activity. Therefore, MOR-1 and MOR-1X expressing cells display distinct differences in Bax proteins expression, which may be due to disparate activation of upstream signaling kinases, RSK1/2 and ERK1/2.

Investigation of autophagic mechanisms did not show significant differences between MOR-1 and MOR-1X signaling as neither receptor significantly altered the expression of autophagic protein markers (Figure 4B–D). Specifically, the expression of Beclin-1, an essential protein component of autophagosome assembly, was not significantly altered by chronic, high dose morphine treatment in untransfected HEK293 cells (Figure 4B, lanes 1–2; Figure 4D, lanes 1–2), vector-transfected cells (Figure 4B, lanes 3–4; Figure 4D, lanes 3–4), pCMV6-MOR-1-transfected cells (Figure 4B, lanes 5–6; Figure 4D, lanes 5–6) or pCMV6-MOR-1X-transfected cells (Figure 4B, lanes 7–8; Figure 4D, lanes 7–8) as well as in untransfected cells treated with 1mM CdCl₂ for 1 hour (Figure 4B, lane 9; Figure 4D, lane 9) or serum starved in HBSS media and treated with 5nM Baf for 4 hours (Figure 4B, lane 10; Figure 4D, lane 10). Expression of LC3-I and LC3-II proteins, which specifically localize to forming and newly formed autophagosomes, were not significantly different between untransfected cells (Figure 4D, lanes 1–2), vector-transfected cells (Figure 4D,

lanes 3–4), pCMV6-MOR-1-transfected cells (Figure 4D, lanes 5–6) and pCMV6-MOR-1X-transfected cells (Figure 4D, lanes 7–8), although untransfected cells treated with 1mM CdCl₂ for 1 hour (Figure 4D, lane 9) or serum starved in HBSS media and treated with 5nM Baf for 4 hours (Figure 4D, lane 10) did exhibit an increased expression in LC3-II protein. Accordingly, the ratio of LC3-I to LC3-II, used as a marker for LC3 conversion during autophagosome formation, was not significantly different between morphine treatment conditions (Figure 4C, lanes 1–8), but was significantly decreased in untransfected cells treated with 1mM CdCl₂ for 1 hour (Figure 4C, lane 9) or serum starved in HBSS media and treated with 5nM Baf for 4 hours (Figure 4C, lane 10), indicating increased autophagosome production. Therefore, while autophagy was clearly utilized by HEK293 cells under stress conditions, neither MOR-1 nor MOR-1X signaling significantly altered autophagic mechanisms.

MOR-1X signaling reduces mitochondrial dehydrogenase activity but not viability

The ERK/RSK signaling pathway regulates cell-cycle progression, proliferation, metabolism, and viability through numerous downstream effectors (Anjum and Blenis, 2008; Gehart et al., 2010; Hauge and Frodin, 2006; Romeo et al., 2011). Given that MOR-1 and MOR-1X expressing cells exhibit unique regulation of phosphorylated ERK1/2, phosphorylated RSK1/2, and Bax protein expression, it is likely that the activity of these receptors exhibit divergent regulation of intrinsic signaling mechanisms of apoptosis, cellular metabolism, and cellular viability. Accordingly, early and late stage apoptosis was determined using Annexin/7-AAD staining. Our results show that MOR-1 and MOR-1X expressing cells do not exhibit significant differences in the establishment of either early or late stage apoptosis constitutively or with chronic, high dose morphine treatment (Figure 4F).

Effects on cellular metabolic activity were next determined using an MTT assay that measures the reduction of the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to insoluble formazan, a process dependent on the activity of the mitochondrial dehydrogenase enzyme. Results found that, relative to the untreated, untransfected control, nearly every treatment condition slightly, but significantly, altered mitochondrial dehydrogenase activity (data not shown). Therefore, significant changes in cellular metabolic activity due to opioid receptor activity were determined relative to untreated, vector-transfected cells. Accordingly, untransfected cells serum starved in HBSS media for 4 hours (Figure 4E, lane 3), serum starved in HBSS media and treated with 5nM Baf for 4 hours (Figure 4E, lane 4), or treated with 1mM CdCl₂ for 1 hour (Figure 4E, lane 5) exhibited significantly reduced mitochondrial dehydrogenase activity. Conversely, mitochondrial dehydrogenase activity was not significantly altered in pCMV6-MOR-1-transfected cells (Figure 4E, lane 8) or pCMV6-MOR-1X-transfected cells (Figure 4E, lane 10). Likewise, treatment with chronic, high dose morphine did not significantly alter mitochondrial dehydrogenase activity in vector-transfected cells (Figure 4E, lane 7) or pCMV6-MOR-1-transfected cells (Figure 4E, lane 9); however, mitochondrial dehydrogenase activity was significantly reduced in pCMV6-MOR-1X-transfected cells following chronic, high dose morphine treatment (Figure 4E, lane 11), further highlighting the functional differences between MOR-1 and MOR-1X.

Discussion

The existence of multiple, ligand specific opioid receptors has been hypothesized for decades due to the unique pharmacological profiles of various opioids. Accordingly, four opioid receptor subtypes have been identified, the KOR, the DOR, the MOR, and the ORL1. These four receptors are highly homologous and are encoded by distinct genes, which are similarly homologous, suggesting their evolution from a common ancestral gene (Dreborg et al., 2008; Waldhoer et al., 2004; Wei and Loh, 2011). However, despite the identification of four opioid receptor subtypes, the binding profiles of opioid compounds do not correlate with the pharmacological profiles suggested by a four-receptor subtype model, raising the question as to how four opioid receptor-encoding genes generate a complex pharmacological profile that suggests additional receptor subtypes. As such, epigenetic, transcriptional, and post-transcriptional mechanisms that generate multiple opioid receptors from individual opioid genes have been identified, most notably constitutive and alternative splicing mechanisms, which selectively remove intronic and exonic regions to generate various isoforms (Hui, 2009; Keren et al., 2010; Regan et al., 2011). Multiple physiological and environmental stimuli are known to alter constitutive and alternative splicing. Accordingly, chronic use and abuse of opioids such as methadone and morphine has been found to alter splicing patterns of the MOR (Verzillo et al., 2014; Voussooghi et al., 2009; Xu et al., 2015). The current study confirms and expands upon these previous findings, as our results demonstrate that morphine treatment significantly increases MOR-1X mRNA expression in a human cell-line model of dopaminergic neurons (Figure 1).

Both constitutive and alternative splicing of pre-mRNA transcripts is initiated by the spliceosome, a large, fluid ribonucleoprotein complex comprised of various snRNPs and numerous non-snRNP splicing factors (Hui, 2009; Keren et al., 2010). These trans-acting factors interact with cis-acting elements, such as 3' and 5' splice sites and exon splicing enhancers and silencers, within the pre-mRNA template to selectively incorporate or exclude intronic and exonic regions in the mature mRNA. Generation of mRNA isoforms via alternative splicing events is determined by the relative activity of and interaction between cis- and trans-acting factors. Accordingly, splicing specificity is determined primarily through the modification of trans-acting spliceosome proteins, specifically SR proteins and hnRNPs, via phosphorylation as well as the increased expression of spliceosome proteins both overall and relative to competing factors (Caceres et al., 1994; Fu, 1995; Stamm, 2008). The current study is the first to suggest that morphine-mediated regulation of OPRM1 splicing specificity utilizes this mechanism, as our results show that ASF/SF2 protein was significantly up-regulated by morphine treatment (Figure 2A) in a manner that correlated with that seen for morphine-mediated increases in MOR-1X mRNA expression (Figure 1). This suggests that the mechanism through which morphine regulates MOR-1X mRNA expression in SH-SY5Y cells involves modulation in ASF/SF2 expression. This proposed mechanism is not entirely unexpected and is supported by the fact that ASF/SF2 facilitates the selection of proximal 5' splice sites in a concentration-dependent manner (Krainer et al., 1990) and the 5' splice site of alternative exon X is proximal to that of the constitutive exon 4 in the MOR pre-mRNA transcript. Therefore, it is likely that levels of ASF/SF2 constitutively expressed in SH-SY5Y cells are not sufficient to readily

promote U1 snRNP recruitment and spliceosome assembly around the intrinsically weak exon X 5' splice site, but as ASF/SF2 levels are increased with morphine treatment, selection of the exon X 5' splice site is favored. The mechanism by which morphine regulates ASF/SF2 expression is still unclear. Our results suggest that this mechanism is independent of SFRS1 promoter activity (Figure 2B–C); however, a small, but statistically insignificant, dose-dependent increase in the activity of the -400 SFRS1 promoter region as well as a slight increase in ASF/SF2 mRNA were observed and, as such, more sensitive measures may elucidate mild transcriptional regulatory mechanisms. It is also possible that morphine-mediated up-regulation of ASF/SF2 protein expression involves some level of translational regulation, as previous studies have found that internal translation contributes to morphine-mediated increases in another auxiliary splicing factor, hnRNP K (Lee et al., 2014).

The alternatively spliced isoform MOR-1X is generated through alternative splicing mechanisms that replace the terminal exon of MOR-1 with an alternative exon cassette (Pan et al., 2003), resulting in both receptors being 99% conserved overall and containing identical N-terminal, loop, and transmembrane domains as well as a proximal portion of the C-terminal domain, all of which contain conserved functional motifs, including the DRY and TREHPS motifs, essential for GPCR- and MOR-specific signaling (Figure 3A–B). As such, any functional differences between MOR-1 and MOR-1X will likely be attributed to the unique distal portion of the C-terminal domain, a region encoded by the terminal exon of both MOR-1 and MOR-1X and the site of phosphorylation reactions essential for initiating conformational changes and GPCR signal transduction. Examination of the predicted primary sequence of this unique region showed that while the distal portion of the MOR-1 C-terminal domain does not contain any identifiable phosphorylation motifs (Figure 3A), the distal portion of the MOR-1X C-terminal domain contains multiple phosphorylation motifs, including two PKA phosphorylation sites and a second copy of the opioid receptor-specific agonist-induced phosphorylation motif (Figure 3B). Variations in the C-terminal domain, such as those observed in the current study, are likely to alter intracellular signaling and constitutive receptor activity, as these regions facilitate the selective recruitment of GPCR interacting proteins, including G proteins, receptor kinases, small GTPases, and β -arrestins, and the subsequent palmitoylation, phosphorylation, signal transduction, internalization, trafficking, and down-regulation or recycling of the receptor (Markovic and Challiss, 2009; Surratt and Adams, 2005). Investigation of signaling differences, specifically within MAPK signaling cascades, in the MOR-null cell line HEK293 found that MOR-1 and MOR-1X have distinct effects on the phosphorylation of ERK1/2 and RSK1/2 both constitutively and following morphine treatment (Figure 3C–G). While it would have been helpful to verify these distinct signaling differences in a more relevant cell type, such as the dopaminergic neuron model cell line SH-SY5Y, robust expression of several MOR isoforms in this cell line makes it impossible to distinguish agonist-mediated signaling of any single isoform, further highlighting the need for reliable MOR-null neuronal models.

The exact mechanisms by which MOR-1 and MOR-1X distinctly alter MAPK signaling is unclear, although differences between the C-terminal domains of MOR-1 and MOR-1X may independently and selectively recruit interacting proteins, such as GRKs, second messenger protein kinases, RGS proteins, and, most notably, β -arrestins. Selectivity of β -arrestins is

often determined by the receptor C-terminal domain as well as specific GRK- and second messenger-dependent protein kinase-mediated phosphorylation of the receptor (Ahn et al., 2004; Barki-Harrington and Rockman, 2008; Cen et al., 2001; Chavkin et al., 2013; Defea, 2008; DeWire et al., 2007; Gurevich and Gurevich, 2006; Lefkowitz and Shenoy, 2005; Luttrell and Lefkowitz, 2002; Macey et al., 2006). More importantly, the recruitment of β -arrestins represents an important kinetic switch between G protein-dependent and G protein-independent signaling. The initial wave of MAPK signaling stimulated by G protein-dependent activation of second messengers is both rapid and transient, with peak signaling occurring within seconds to minutes, and results in unique MAPK downstream signaling cascades, such as the translocation of ERK1/2 into the nucleus and the activation of ERK1/2 transcriptional pathways. However, displacement of G proteins by β -arrestins interrupts this G protein-dependent signaling and facilitates the construction of β -arrestin signaling complexes, which results in a second wave of unique MAPK downstream signaling cascades that is comparatively delayed in onset but more persistent than those initiated by G proteins. Due to the fact that β -arrestin signaling complexes are sequestered in the cytoplasm, these G protein-independent MAPK signaling cascades exhibit enhanced cytoplasmic signaling but diminished nuclear signaling. Accordingly, the formation of β -arrestin/ERK signaling complexes enhances the phosphorylation of cytoplasmic ERK1/2 substrates such as RSK1/2 (Chavkin et al., 2013). Therefore, structural differences between MOR-1 and MOR-1X may alter the recruitment of β -arrestins, both constitutively and following agonist-activation, and subsequently alter the distinct physiological endpoints of MAPK signaling cascades, such as ERK1/2 and RSK1/2, that are temporally and spatially regulated through a two-phase signaling cascade.

The divergent regulation of ERK/RSK signaling cascades by MOR-1 and MOR-1X may have significant functional consequences in opioid pharmacology as RSK2 signaling contributes to morphine-mediated analgesia (Darcq et al., 2012). Furthermore, ERK/RSK signaling is implicated in multiple cell processes, including cellular metabolism and viability. The current study found that cellular viability was not significantly different between cells expressing MOR-1 or MOR-1X, both constitutively and following chronic, high dose morphine treatment (Figure 4F); however, expression of the pro-apoptotic protein Bax was significantly reduced by the constitutive activity of the MOR-1X receptor (Figure 4A). Furthermore, cellular metabolic activity, as determined by mitochondrial dehydrogenase activity, was significantly decreased in MOR-1X expressing cells when treated with chronic, high dose morphine (Figure 4E). The potential mechanisms through which MOR-1X mediate these unique effects are numerous. RSK-mediated regulation of cellular viability is primarily due to phosphorylation and/or inactivation of pro-apoptotic proteins, as observed here; however, RSK1/2, as well as a related protein MSK2, may also utilize mechanisms involving downstream transcriptional factors like p53 and CREB, which subsequently direct the synthesis of pro- and anti-apoptotic Bcl-2 family member proteins, respectively (Anjum and Blenis, 2008; Chavkin et al., 2013; Hauge and Frodin, 2006; Krishna and Narang, 2008). Likewise, increasing evidence suggests that cellular metabolism is regulated by MAPKs, such as ERK1/2 and p90 RSK, through complex signaling pathways mediating substrate phosphorylation and transcriptional regulation (Gehart et al., 2010). Therefore, decreased Bax expression and cellular metabolic activity in MOR-1X

expressing cells, constitutively and following morphine treatment, respectively, is most likely due to dynamic and complex interactions between independent ERK/RSK and MSK2 signaling mechanisms involving direct phosphorylation of downstream substrates as well as transcriptional regulation.

While the current study, as well as previous studies, have begun to identify the roles of individual opioid receptor isoforms in opioid signaling and pharmacology, it remains difficult to accurately predict the potential physiological effects and clinical relevance of any one isoform. Given that MOR isoforms exhibit unique ligand binding, desensitization, internalization, and recycling characteristics, it can be speculated that effects due to shifting isoform expression profiles will include variations in opioid sensitivity, tolerance, addiction, and withdrawal on both a cellular and physiological level. Furthermore, differences in signaling cascades that mediate opioid analgesia, such as the unique RSK signaling mediated by MOR-1X found in the current study, may be responsible for the variation in analgesia induced by different opioid agonists while differences in signaling cascades that mediate cell viability, such as the unique Bax expression and mitochondrial dehydrogenase activity of MOR-1X expressing cells observed here, may result in either the exacerbation or attenuation of pathological conditions associated with opioid use. As such, the revelation here and in previous studies that opioids autoregulate MOR splicing, potentially through the modulation of auxiliary splicing factors such as ASF/SF2, not only provides an explanation for the observed variation in isoform expression profiles among opioid exposed populations, particularly those with a history of opioid abuse, but also suggests that opioid receptor isoform expression, and subsequently opioid pharmacology, is dynamic and can be manipulated by both endogenous and exogenous factors. Accordingly, the novel regulatory mechanism of opioid receptor splicing involving ASF/SF2 presented here has extensive clinical potential, as therapeutic compounds targeting the splicing activity of ASF/SF2, and other auxiliary splicing factors, could be used to manipulate MOR splicing in order to generate a more favorable receptor profile and subsequent clinical responses to opioids and opioid withdrawal. This is all in addition to the cellular, physiological, and clinical potential opioids have in mediating alternative splicing of other cellular proteins, as ASF/SF2 is considered a general splicing factor and, therefore, opioid-mediated fluctuations in ASF/SF2 expression are likely to alter the splicing patterns of multiple proteins.

In summary, the current study supports previous findings that opioids such as morphine mediate changes in MOR splicing patterns and is the first to propose a mechanism through which this is regulated, specifically involving the regulation of the auxiliary splicing factor ASF/SF2. Furthermore, this mechanism of morphine-mediated ASF/SF2 regulation does not appear to involve transcriptional regulation of the ASF/SF2 encoding gene, SFRS1, and therefore supports previous studies that identified morphine-mediated translational regulation of auxiliary splicing factors. Structural comparison between MOR-1 and MOR-1X suggests that MOR-1X has a greater potential for phosphorylation due to additional phosphorylation motifs within the unique portion of the C-terminal tail while functional comparison identified distinct differences in phosphorylated ERK1/2, RSK1/2, and pro-apoptotic Bax protein expression, but not in the expression of autophagic proteins Beclin-1 or LC3. Although this did not result in significantly altered cellular viability, cellular metabolism was significantly, albeit slightly, reduced in MOR-1X expressing cells.

Therefore, while the current study supports previous findings that morphine regulates MOR splicing, and that individual MOR isoforms have functional significance, additional studies are needed to further confirm and characterize these processes.

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Figure 1.

Figure 2.

Figure 3.

Figure 4.